Effects of ocean acidification on trace element accumulation in the early-life stages of squid Loligo vulgaris

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Abstract

The anthropogenic release of carbon dioxide (CO_2) into the atmosphere leads to an increase in the CO_2 partial pressure (pCO_2) in the ocean, which may reach 950 µatm by the end of the 21st century. The resulting hypercapnia (high pCO_2) and decreasing pH ("ocean acidification") are expected to have appreciable effects on water-breathing organisms, especially on their early-life stages. For organisms like squid that lay their eggs in coastal areas where the embryo and then paralarva are also exposed to metal contamination, there is a need for information on how ocean acidification may influence trace element bioaccumulation during their development. In this study, we investigated the effects of enhanced levels of pCO_2 (380, 850 and 1500 µatm corresponding to pH_T of 8.1, 7.85 and 7.60) on the accumulation of dissolved ^{110m}Ag, ^{109}Cd, ^{57}Co, ^{203}Hg, ^{54}Mn and ^{65}Zn radiotracers in the whole egg strand and in the different compartments of the egg of *Loligo vulgaris* during the embryonic development and also in hatchlings during their first days of paralarval life. Retention properties of the eggshell for ^{110m}Ag, ^{203}Hg and ^{65}Zn were affected by the pCO_2 treatments. In the embryo, increasing seawater pCO_2 enhanced the uptake of both ^{110m}Ag and ^{65}Zn while ^{203}Hg showed a minimum concentration factor (CF) at the intermediate pCO_2. ^{65}Zn incorporation in statoliths also increased with increasing pCO_2. Conversely, uptake of ^{109}Cd and ^{54}Mn in the embryo decreased as a function of increasing pCO_2. Only the accumulation of ^{57}Co in embryos was not affected by increasing pCO_2. In paralarvae, the CF of ^{110m}Ag increased with increasing pCO_2, whereas the ^{57}Co CF was reduced at the highest pCO_2 and ^{203}Hg showed a maximal uptake rate at the intermediate pCO_2. ^{54}Mn and ^{65}Zn accumulation in paralarvae were not significantly modified by hypercapnic conditions. Our results suggest a combined effect of pH on the adsorption and protective properties of the eggshell and of hypercapnia on the metabolism of embryo and paralarvae, both causing changes to the accumulation of metals in the tissues of *L. vulgaris*.

Keyword: cephalopod, metal, CO_2, embryo, paralarvae, eggshell

Introduction

Rising atmospheric carbon dioxide (CO_2) levels, driven by fossil fuel combustion, cement production and changing land use are generating major changes in the chemistry of seawater. The global atmospheric concentration of CO_2 has increased from a pre-industrial value of approximately 280 ppmv to 379 ppmv in 2005 (IPCC, 2007) and is projected to further increase in the coming decades. It is expected to exceed 500 ppmv by year 2050 and could reach 950-1020 ppmv by the end of the century (Orr, in press). By air-sea equilibration, the uptake of anthropogenic CO_2 by the oceanic surface waters is consecutively expected to increase (Sabine et al., 2004). This will cause major shifts in the ocean carbonate chemistry, i.e. increased concentrations of dissolved inorganic carbon and bicarbonate ions, decreased carbonate concentration, and pH is expected to decline by 0.2–0.4 units, a phenomenon called "ocean acidification" (Caldeira and Wickett, 2005).

The consequences of ocean acidification for marine biota could be wide ranging. Calcification is certainly the process that has been investigated the most to date. The decrease in the availability of carbonate is expected to lead to reductions in the calcification rate of many organisms that precipitate shells and skeleton (*e.g.* Gattuso et al., 1998, 1999; Doney et al., 2009) and acidified seawater could become corrosive to these $CaCO_3$ structures (Orr et al. 2005). Impacts of dissolved CO_2 on the biology of water-breathers are known to alter survival rates (Talmage and Gobler, 2010), development (Kurihara, 2008; Dupont and Thorndyke, 2009), growth rate (Findlay et al., 2010), immune response (Bibby et al., 2008), feeding rate (Dupont and Thorndyke, 2009), sensory capacities (Munday et al., 2009) and metabolism (Lannig et al., 2010). Some of the physiological responses are considered to be consequences of ionic and acid-base balance disturbances (Deigweiher et al., 2008; Pörtner, 2008; Pörtner and Farrell, 2009). However, some organisms such as fish, crustacean, or echinoderms are not impaired by hypercapnia (high pCO_2) or exhibit unexpected responses such as increased calcification or growth rates (*e.g.* Melzner et al., 2009; Dupont et al., 2010).

Cephalopods have been considered to be particularly vulnerable to ocean acidification due to peculiar aspects of their physiology. They are active coastal invertebrates (O'Dor and Webber, 1986) enabling them, especially squid, to compete with fish for similar ecological niches (Packard, 1972; Arkhipkin and Laptikhovsky, 2010). But, cephalopods' aerobic performance is sustained by the highly pH-dependent affinity (*i.e.* Bohr effect) of their O_2-carrying hemocyanin that delivers O_2 to their tissues and cells (Bridges, 1994). Thus, changes in extra- and intracellular pH resulting from increasing environmental pCO_2 are expected to

have major impacts on the physiological performances of cephalopods (Pörtner and Zielinski, 1998; Pörtner, 2008). A recent report has revealed, however, that the cuttlefish, *Sepia officinalis*, exhibits both higher rates of growth and calcification at 6000 µatm of pCO_2 (Gutowska et al., 2010a) and compensates for hypercapnia-induced extracellular acidosis with no significant increase in energy expenditure (Gutowska et al., 2010b). Nevertheless, invertebrate embryonic and larval stages are considered to be very sensitive in regard to ocean acidification (Dupont et al., 2010) while their respiratory system is still immature (Pörtner and Farrell, 2008). Moreover, cephalopods lay their eggs in shallow waters prior to their death (Worms, 1983) that results in their early-life stages being exposed to coastal metallic contamination. As changes in pH and carbonate chemistry are also predicted to alter the chemical speciation of trace elements, ocean acidification will possibly modify their bioavailability to marine biota (Byrne et al., 1988). Nowadays, there is little information on how the cephalopod early-life stages will respond to ocean acidification in combination with other stressors such as contaminants, that are predicted to also increase in coastal waters (Harley et al., 2006).

In cuttlefish, the eggshell surrounding the embryo acts as a protective barrier that limits or hinders the incorporation of metals into the organism during its initial developmental stages, and with a permeability that is element-specific (Bustamante et al., 2002, 2004, 2006; Lacoue-Labarthe et al., 2008). However, Lacoue-Labarthe et al. (2009a) observed that increased pCO_2 strongly affects Ag, Cd and Zn uptake in embryos, probably through pH-induced changes in the permeability of the eggshell. Compared to quite large eggs of cuttlefish encapsulated in hard shells, small squid eggs (reduced in size by 1 order of magnitude) enveloped in a soft and gelatinous shell produce active swimming hatchlings (Boletzky, 1986, Nixon and Mangold, 1998), and, as recently observed, showed higher metal bioaccumulation capacities (Lacoue-Labarthe et al., sbm). Such differences in egg structure may well modify the effects that ocean acidification would have on trace elements uptake in the early-life stages of squid. To test this hypothesis, this radiotracer study determined the effect of hypercapnia on the accumulation biokinetics and distribution of ^{110m}Ag, ^{109}Cd, ^{57}Co, ^{203}Hg, ^{54}Mn and ^{65}Zn in the early-life stages of *L. vulgaris* that encompassed the developmental phases from the newly spawned encapsulated embryos through to the 52 hours eggshell-free “paralarvae”.

Materials and Methods

1. Organisms, radiotracers and experimental procedures

Egg strand experiment

Egg masses of the squid *Loligo vulgaris* (1-2 d post spawning) were netted at 50 to 200 m water depth in front of Monaco in June 2009. These egg strands (n = 30) were immediately separated and haphazardly assigned to three 5 l plastic bottles (one bottle per treatment) filled with filtered (0.45 µm) and UV-sterilized Mediterranean seawater. Seawater was constantly aerated and the light:dark cycle was 12:12. Eggs were maintained under regulated conditions of pH: one bottle was maintained at ambient pH_T (8.10; pH_T is the pH expressed on the total scale) whereas the other two were maintained at two pH_T levels (7.85 and 7.60). The pH_T levels were chosen according to values predicted to occur by the end of this century according to various scenarios for the trajectories of carbon emissions [(Orr et al., 2005: 7.85 (pCO_2 = 850 µatm), 7.60 (pCO_2 = 1500 µatm)]. Once stable pH was reached (see below), radiotracers were added to each bottle and the trace metal uptake kinetics were determined, as described below. Seawater was renewed daily with sterilized, filtered and pH-adjusted Mediterranean seawater during the first three days and then every second or third day in order to maintain good water quality and radiotracers concentrations as constant as possible. Bottles were changed and cleaned at each seawater renewal to prevent any "bottle" effect due to the development of different biomasses due to the accumulation of detritus such as fragments of eggshell layers, or due to bacterial proliferation, which could affect the metabolism of eggs or the bioavailability of trace elements.

Paralarvae experiment

Egg strands (n = 15) from the initial egg masses were maintained under controlled conditions until the first larvae hatched. At this time, egg strands were placed in 5 l bottles filled with filtered and sterilized seawater and controlled pH until an adequate number of paralarvae for the experiment were obtained, *i.e.* 100 for each experimental condition. Radiotracers were then introduced into each bottle and the metal uptake kinetics were monitored during 52 d, as described below.

CO_2 incubations

Water bottle pH values were maintained within ± 0.05 unit with a pH-stat system (IKS, Karlsbad) that added pure CO_2 into the bottles that were continuously aerated with CO_2-free

air. The pH_{NBS} (National Bureau of Standard scale) values were logged every 10 min and were converted from the NBS scale to the total scale (pH_T) using point pH measurements on both scales performed every 2 days. The pH values were measured using a pH meter (Metrohm, 826 pH mobile) with a glass electrode (Metrohm, electrode plus) that was calibrated on the total scale using Tris/HCl and 2-aminopyridine/HCl buffer solutions with a salinity of 38 and prepared according to Dickson et al. (2007). Total alkalinity (TA) changes between two seawater renewals were assessed in a control (*i.e.* radiotracers-free) bottle containing similar egg strand masses or the same number of paralarvae and maintained at ambient pH_T (*ca*. 8.1) and temperature (16°C). TA was measured on seawater samples filtered through 0.45 µm membranes that were immediately poisoned with mercuric chloride and stored in a cool dark place pending analysis. Seawater was sampled just before and after seawater renewal. TA was determined potentiometrically using a home-made titration system with an Orion 8103SC pH electrode calibrated on the NBS scale and a computer-driven Metrohm 665 Dosimat titrator. TA was calculated using a Gran function applied to pH values ranging from 3.5 to 3.0 as described by Dickson et al. (2007). The pCO_2 was determined from pH_T and total alkalinity using the R package seacarb (Lavigne and Gattuso, 2010).

2. Radiotracers exposure

Water bottles were spiked with radioactive ^{110m}Ag (1 kBq l^{-1}), ^{109}Cd (1.5 kBq l^{-1}), ^{57}Co (1 kBq l^{-1}), ^{203}Hg (1 kBq l^{-1}), ^{54}Mn (1 kBq l^{-1}) and ^{65}Zn (1 kBq l^{-1}). These activities corresponded to an addition of 14, 11, 92 and 0.9 pmol L^{-1} of Ag, Cd, Co and Zn, respectively, to the natural concentrations present in the filtered Mediterranean seawater. Specific activities of ^{54}Mn and ^{203}Hg sources were not available according to the provider. These additions of metals per spike were one to five orders of magnitude lower than the natural concentrations of metals reported in seawater (Bruland, 1983). Radiotracers were purchased from Amersham, UK (^{110m}Ag, ^{109}Cd and ^{57}Co) and Isotope Product Laboratory, USA (^{54}Mn and ^{65}Zn): ^{110m}Ag [as $^{110m}AgNO_3$; T½ = 250 d], ^{109}Cd [as $^{109}CdCl_2$; T½ = 464 d], ^{57}Co [as $^{57}CoCl_2$; T½ = 272 d], ^{203}Hg [as $^{203}HgCl_2$; T½ = 46.6 d], ^{54}Mn [as $^{54}MnCl_2$; T½ = 312 d] and ^{65}Zn [as $^{65}ZnCl_2$; T½ = 244 d]. Stock solutions were prepared in 0.1 N nitric acid (^{110m}Ag), in 0.1 N chloridric acid (^{109}Cd, ^{57}Co, ^{54}Mn and ^{65}Zn, respectively) and in H_2O (^{203}Hg). Spikes volume was typically 5 µL.

For each treatment, radiotracers spikes were renewed after each water renewal, *i.e.* daily, during the first three days and then every second or third day to maintain radiotracer concentrations as stable as possible. Radiotracers activities in seawater were checked before

and after each water renewal in order to determine the time-integrated radiotracers activities, *i.e.* the mean value of all measurements performed over the time period considered (Metian et al., 2009).

Egg experiment: at different time intervals the six radionuclides activities were counted in the same 4 labelled egg strands per bottle during the total experimental exposure. After 16 d and 26 d of development (respectively at stage 25-26 and 29-30), 3 additional egg strands from each experimental condition were counted and dissected to determine the radiotracer distribution among the external (*i.e.* nidamental eggshell) and internal (*i.e.* oviducal eggshell including the chorion) eggshell parts, the vitellus, the embryo and the perivitelline fluid. Statoliths were removed from pre-hatched embryos (26 d) and pooled for activity measurement according to their rearing conditions (n = 95, 126 and 115 at 1500, 850 and 380 µatm, respectively). Additionally, statoliths of late-stage embryos that had been maintained at the same pCO_2 treatments, but not exposed to radiotracers, were extracted for surface area measurement using ImageJ® software. These measurements provided an estimate of their size at the end of development in each treatment (n = 21, 22 and 15 at 1500, 850 and 380 µatm, respectively).

Paralarvae experiment: 5 groups of paralarvae (n = 5 -10 in order to facilitate radiotracer activities detection) were sampled from each pCO_2 condition after 6, 28 and 52 h of exposure, rapidly rinsed with unlabelled seawater, weighted and counted as described below.

3. Radioanalyses and data treatments

Radioactivities were measured using a high-resolution γ-spectrometry system consisting of four coaxial Germanium (N- or P-type) detectors (EGNC 33-195-R, Canberra® and Eurysis®) connected to a multi-channel analyzer and a computer equipped with a spectra analysis software (Interwinner® 6). The detectors were calibrated with an appropriate standard for each counting geometry that was used and measurements were corrected for background and physical decay of the radiotracers. Counting times were adapted to obtain relative propagated errors less than 5%. They ranged from 10 to 30 min for whole eggs and from 10 min to 24 h for the dissected egg compartments.

Uptake of ^{110m}Ag, ^{109}Cd, ^{57}Co, ^{203}Hg, ^{54}Mn and ^{65}Zn was expressed as changes in concentration factors (CF), which is the ratio between radiotracer activity in the egg strand, egg compartment or paralarvae (Bq g^{-1}) and the time-integrated activity in seawater (Bq g^{-1}; Metian et al., 2009). This unit-less term expresses the efficiency of an organism (or a

biological compartment) to accumulate and concentrate an element from seawater after a fixed time of exposure.

Uptake kinetics were best described using either a linear equation (Eq. 1), a saturation exponential equation (Eq. 2), or a combined equation (exponential and linear) (Eq. 3):

$CF_t = k_u\, t$ (Eq. 1)

$CF_t = CF_{ss}\,(1\text{-}e^{-ke\, t})$ (Eq. 2)

$CF_t = CF_{ss}\,(1\text{-}e^{-ke1\, t}) + k_{u2}\, t$ with $CF_{ss} = k_{u1} / k_{e1}$ (Eq. 3)

where CF_t and CF_{ss} are the concentration factors at time t (d) and at steady-state, and k_e and k_u are the biological depuration and uptake rate constants (d^{-1}) (Whicker and Schultz, 1982).

Constants for the best fitting uptake kinetic equations (decision based on ANOVA tables for two fitted model objects) as well as their statistics of variability were estimated by iterative adjustment of the models using the nls or lm curve-fitting routine in R (R Development Core Team, 2008). F-tests were used to determine whether pCO_2 exposure influenced the uptake rate (k_u) of radiotracers or the CF_{ss} reached at the steady-state equilibrium. A Kruskal-Wallis test was applied to determine the differences in the radiotracers CF reached in embryo and eggshell at the late embryonic stages (stage 29-30) among the different pCO_2 treatments. The level of significance for statistical analyses was always set at $\alpha = 0.05$. Results are reported as means ± s.d.

Results

1. Carbonate chemistry during the incubations

During embryonic development, pH_T was maintained at 7.57 ± 0.03, 7.79 ± 0.05, and 8.08 ± 0.04 at a temperature of 16.1 ± 0.4°C. Mean TA of renewed seawater was 2.521 ± 0.005 mmol kg^{-1} (range of 2.512 to 2.528 mmol kg^{-1}). It changed by 0.005 to 0.050 mmol kg^{-1} between two seawater renewals (TA range of 2497 to 2575 mmol kg^{-1}). These conditions correspond to the following ranges of pCO_2; 1470 to 1507, 846 to 868 and 392 to 401 µatm, respectively in the three treatments. Squid paralarvae were maintained at pH_T 7.55 ± 0.02, 7.90 ± 0.02 and 8.12 ± 0.02 at a temperature of 15.8 ± 0.3°C. The mean TA of renewed seawater was 2.526 ± 0.003 mmol kg^{-1}. It changed by 0.095 to 0.105 mmol kg^{-1} between two seawater renewals. These conditions correspond to ranges of pCO_2 of 1550 to 1613 µatm, 639 to 666 µatm and 353 to 368 µatm, respectively in the three treatments.

2. Uptake kinetics in the egg strands and embryos

The uptake kinetics in whole egg strands showed contrasting patterns among radiotracers (Fig. 1; Table 1). Accumulation of ^{109}Cd, ^{203}Hg, ^{54}Mn and ^{65}Zn were best fitted by a saturation exponential model at every pCO_2 levels. At steady state equilibrium, CF_{ss} of ^{109}Cd, ^{54}Mn and ^{65}Zn were significantly lower at pCO_2 850 and 1500 µatm than at 380 µatm (^{109}Cd CF_{ss}: 37 ± 4 and 29 ± 2 < 77 ± 5; ^{54}Mn CF_{ss}: 50 ± 3 and 78 ± 6 < 113 ± 5; ^{65}Zn CF_{ss}: 201 ± 18 and 208 ± 10 < 299 ± 16 at 850 and 1500 < 380 µatm, respectively). Surprisingly, ^{203}Hg uptake efficiency was maximal at the 850 µatm (^{203}Hg CF_{ss}: 371 ± 9 > 334 ± 8 and 292 ± 8 at pCO_2 850 > 1500 and 380 µatm, respectively). For each of the pCO_2 conditions, ^{110m}Ag accumulation showed a two-step process (exponential + linear combined equation) with a rapid adsorption and/or absorption (*e.g.* k_{u1} = 40 d^{-1} at 380 µatm) followed by a slower uptake phase (*e.g.* k_{u2} = 0.05 d^{-1} at 380 µatm). Whereas pCO_2 did not influence k_{u1}, higher k_{u2} was observed at 850 µatm, (0.08 d^{-1} at 850 µatm > 0.05 d^{-1} at 380 and 1500 µatm), indicating that only the second Ag binding process was influenced by pCO_2. Finally, ^{57}Co displayed linear kinetics and the uptake rates were unaffected by pCO_2 (k_u: 4.98, 4.95, 4.49 d^{-1} at 380, 850 and 1500 µatm, respectively; $p > 0.05$). Accumulation of this element in the squid egg masses was therefore at a constant linear rate.

With regard to the CF's in the squid embryo that developed under different pCO_2 conditions (Fig. 2), and at two periods of development, the accumulation of ^{110m}Ag was significantly higher at the highest pCO_2 level than at the two lower exposure levels, irrespective of the development time (*i.e.* after 16 and 26 d of development). Similarly, the lower the seawater pH, the more that ^{65}Zn was accumulated in the tissues of embryos at the end of their development, with ^{65}Zn CFs that were 1.2- and 1.4-fold higher at pH_T 7.85 and 7.60 than at normal pH_T, respectively. This result contrasts with the maximal ^{65}Zn CF values in the embryo at 16 d at pH_T 7.85. In contrast, the ^{109}Cd and ^{54}Mn CFs in the embryo were reduced by decreasing pH_T. No significant effect of pCO_2 was observed on ^{57}Co uptake. Nevertheless, the ^{57}Co concentration factor was very variable at the end of development (CF_{Co} = 317 ± 377) in embryos that had been maintained under control pCO_2 level (see details below, section 3). Finally, the ^{203}Hg CF was lowest at pH_T 7.85 in embryos that were sampled a few hours before hatching, whereas no pCO_2-effect on ^{203}Hg accumulation was observed after 16 d of embryonic development.

3. Radiotracers distribution and uptake in egg strand compartments

Radiotracer distributions among different egg compartments at the end of development (Table 2) show that pCO_2 had little effect for Cd, Co, Hg and Mn, which remained mainly associated (> 90% of the total amount) with the eggshell. More precisely, ^{54}Mn, ^{203}Hg, and, to a lesser extent, ^{109}Cd were mainly bound to the external layers of the eggshell (Table 2; 92%, 80% and 55% at normal pH, respectively) which is composed of the secretions of the nidamental gland. Whole eggshell CF values for ^{109}Cd and ^{54}Mn decreased with increasing pCO_2, whereas the highest ^{203}Hg CF in eggshell was found at a pCO_2 850 µatm, a CO_2 level where the Hg concentration in the embryos was the lowest.

Interestingly, ^{57}Co was predominantly found in the internal eggshell parts, although it also showed high standard deviation values. Actually, in one of the three egg strands analyzed, an 8-fold higher Co accumulation in embryos was noted (CF_{Co} = 834 ± 142 *vs.* 104 ± 84). This was associated with a CF value in the internal eggshell layers that was 4.5-fold lower compared to the concentration found in the other egg strands (CF_{Co} = 83 ± 18 *vs.* 386 ± 53). This result suggests a failure in the Co retention capacities of the internal shells and therefore in the protective role of the chorion against Co penetration.

The proportion of ^{110m}Ag and ^{65}Zn detected in the eggshell was reduced between the normal and the highest pCO_2, from 55% to 37% and from 90% to 70%, respectively whereas it increased in the embryo from 45% to 62% and from 10% to 30%, respectively. These distributions match with the maximum ^{110m}Ag CF value found at 800 µatm (pH_T 7.85) and the decrease of the ^{65}Zn accumulation in the eggshell with increasing pCO_2 (^{110m}Ag CF: 425 ± 102 > 255 ± 74 > 187 ± 58, *i.e.* $CF_{7.85} > CF_{8.10} > CF_{7.60}$ and ^{65}Zn: $CF_{8.10} \approx CF_{7.85} > CF_{7.60}$) occurring concomitantly with the increase of the metal uptake efficiency in the embryo (Table 2; see section 2 above). These results suggest, as observed for ^{203}Hg, that the less the metals were accumulated/retained by the eggshell with increasing pCO_2, the more they were found in the embryos contained within.

4. Zn accumulation in embryo statoliths

Only ^{65}Zn accumulated to a detectable degree in the statoliths of embryos that were sampled and pooled for analysis at the end of the developmental phase. Its load concentration ratio (LCR_{st} in g, corresponding to the activity in one statolith (Bq) normalized by the activity in seawater ($Bq.ml^{-1}$); Lacoue-Labarthe et al., 2008) followed a trend similar to that observed in the whole body of the embryo. It increased with increasing pCO_2, *i.e.* 2.68 x 10^{-3} g, 3.10 x 10^{-3} g and 3.93 x 10^{-3} g at 380, 850 and 1500 µatm, respectively. Moreover, the statoliths were significantly bigger when embryos developed at 1500 µatm and 850 µatm compared to 380

µatm with mean surface areas of 14.27 ± 1.06 mm^2 and 14.53 ± 0.67 mm^2 against 12.91 ± 0.59 mm^2, respectively (ANOVA, $p < 0.001$). However, the mantle length of embryos did not vary significantly between treatments (2.51 ± 0.07 mm, 2.56 ± 0.07 mm and 2.50 ± 0.05 mm at 1500, 800 and 380 µatm, respectively; ANOVA, $p > 0.05$).

5. Uptake kinetics of tracers in the three first days of "paralarval" life

Despite the relatively short experimental exposure (52 h), the ^{110m}Ag and ^{60}Co uptake kinetics were best described by a saturation exponential model ($R^2 = 0.81$), whereas ^{203}Hg, ^{54}Mn and ^{65}Zn kinetics were best fitted by a linear model ($R^2 = 0.90$) (Fig. 3, Table 2). The ^{109}Cd uptake kinetic was not established as recorded activities were below the limit of detection. As found in the embryo, the lower the pH, the higher ^{110m}Ag CF_{ss} accumulation was in the paralarvae, with $CF_{ss\text{-}7.60}$ and $CF_{ss\text{-}7.85}$ that were 1.8- and 1.4-fold higher than $CF_{ss\text{-}8.10}$. In contrast, ^{60}Co CF_{ss} was significantly lower at pH_T 7.60 compared to intermediate and control pHs (Table 2). Similarly to the previous observations on the egg strand, the ^{203}Hg uptake kinetics was enhanced at pH_T 7.85 with higher uptake rate constant compared to those at pH_T 8.10 and 7.60 (Table 2). No significant pH effect was observed on the uptake rate of ^{54}Mn and ^{65}Zn, but ^{65}Zn $CF_{7.85}$ reached at the end of exposure time was significantly higher than $CF_{7.60}$ (Kruskal-Wallis test, $p < 0.05$). Similarly, ^{54}Mn $CF_{7.60}$ was significantly lower than $CF_{8.10}$ after 52 h of exposure (Kruskall-Wallis test: $p < 0.01$), suggesting that increasing pCO_2 could limit the Mn accumulation in the paralarvae.

Discussion

Squid egg strand is characterized by a complex structure in which several small oocytes surrounded by a chorion are united by oviducal jelly, and enveloped by a common sheet of nidamental secretions (Boletzky et al., 1986). As in cuttlefish eggs, some structural changes occur during the embryonic development. The volume, the surface and the weight of the egg strand increase throughout development as a consequence of the swelling (massive entry of water through the eggshell) of the numerous eggs embedded in the same cluster. In cuttlefish eggs, it has been demonstrated that the increased permeability of the egg to some metals is correlated with this swelling process (Lacoue-Labarthe et al., 2008, 2010). In the present study, the uptake of radiotracers in the whole egg strand was expressed in terms of metal concentration factor (CF). The trace elements accumulation was therefore under-

estimated by the rising egg weight that in turn led to a dilution effect (Lacoue-Labarthe et al., 2008). Data were nevertheless expressed in terms of CF so as to compare results obtained on egg strands that were highly variable in terms of length and number of eggs embedded inside.

The effect of acidity on metal toxicity has been well documented, especially in freshwater organisms (*e.g.* Wood, 1992; Carvahlo and Fernandes, 2006). However, to the best of our knowledge, few studies have investigated the impact of increased pCO_2 on metals accumulation and effects on marine organisms (Lacoue-Labarthe et al., 2009a, Pascal et al., 2010). Two aspects of the combined impacts of hypercapnia and metal toxicity on water-breathing animals must be considered. On the one hand, the speciation and, subsequently the bioavailability of metals that form strong complexes with hydroxide and carbonate ions (Byrne, 2002), both of which being expected to decrease in seawater (Feely et al., 2004), might be affected (Shi et al., 2010). Moreover, the increase in the concentration of H^+ could lead to a competition between protons and cationic metals for the binding sites on the biological surface (Millero et al., 2009). On the other hand, seawater pCO_2 might impair the acid-base balance and ion regulation (*e.g.* Pörtner, 2008) and hence disturb active transport or passive diffusion of metals through epithelial membrane (*e.g.* Rainbow, 1997a) and therefore accumulation in tissues.

In a previous study carried out in similar experimental conditions (Lacoue-Labarthe et al., 2009a), speciation modelling has shown that decreased pH_T from 8.10 to 7.60 had a very minor effect on the speciation of Ag, Cd or Zn and their concentration as free metal ions, *i.e.* the bioavailable form of metals, in this experimental water. Through modeling, Millero et al. (2009) showed that metals forming strong chloride complexes (*e.g.* Ag, Cd and Hg) are not affected by decreased pH. Moreover, the predominant free form for Co, Mn and Zn increased by only a few percent (4%, 2% and 13%, respectively) with decreasing pH. Consequently, any observed effect of pH on the accumulation of these six trace elements by squid early-life stages can be regarded as predominantly due to responses of the exposed tissues and/or competition with elevated $[H^+]$ at the cell surface binding sites.

The changes in whole egg metal uptake kinetics with increasing pCO_2 (Fig. 1) can be regarded as an effect of pH on the metal accumulation in the eggshell. As already reported, most of metals (> 90% of the total amount except for Ag ~50 %) is found associated with the eggshell (Lacoue-Labarthe et al., 2008, 2010). This is likely because of the presence of metal binding carboxyl- and sulfhydryl-rich groups (*e.g.* Rainbow, 1997b) in the mucine-rich egg

surface layers (Boletzky et al., 1986). Under acidified conditions, the lowered ^{109}Cd, ^{54}Mn and ^{65}Zn CF_{ss} values in the egg strands (Fig. 1) and in the eggshell (Table 2) suggest that these "Class Borderline" elements, *i.e.* metals exhibiting equal binding-affinity for O or S (Nieboer and Richardson, 1980), are subjected to increased competition with protons for the adsorption on binding sites located on the external eggshell surface. At pH_T 7.85, the highest ^{110m}Ag uptake rate and ^{203}Hg CF_{ss} in egg strand coincide with the maximal accumulation of both metals in the eggshell (Table 2). Ag and Hg are "Class B" elements, *i.e*. with a strong affinity for the sulfhydryl groups, suggesting that pCO_2 at 850 µatm enhanced the binding capacities of the eggshell components for these elements. So it can be hypothesized that ocean acidification affects the metal accumulation in both the eggshell and whole egg strands as a result of competition with H^+ for binding sites to a degree that is dependent on the chemical properties of trace element (Nieboer and Richardson, 1980). Moreover, it should be also pointed out that, in cephalopods, the symbiotic bacterial population embedded in the eggshell nidamental layers (Barbieri et al., 1997) could interfere with the uptake of metals. So far, all the mechanisms involved in the metal accumulation patterns of the eggshell under different pH are not fully understood nor quantified. However, this study clearly shows that elevated pCO_2 modifies the metal binding and retention properties of the eggshell and could therefore affect its shielding properties regarding metal accumulation in embryos (Lacoue-Labarthe et al., 2008, 2009a).

Indeed, the observed metal CFs in the late stage embryo at different pH_T values (Fig. 2) raise the question of the effect of pCO_2 on the protective role of the eggshell and/or on the embryo metabolism that both could modulate the metal uptake in the organism. In this study, two distinct patterns have been found when comparing the metal accumulation in embryo and eggshell at various pCO_2 levels: i) the uptake of ^{109}Cd and ^{54}Mn in the embryo decreased together with the reduced concentration in the eggshell with increasing pCO_2. In contrast, ii) the more the ^{110m}Ag, ^{203}Hg and ^{65}Zn (including also ^{57}Co; see below) accumulated in the eggshell compartment, the lower were the CF values in the embryo.

First, the Cd and Mn showed a limited bioaccumulation in the embryo with increasing pCO_2. In the cephalopod egg, large gradients of partial pressure of O_2 and CO_2 across the eggshell are necessary to drive gas exchanges between seawater and the PVF (Cronin and Seymour, 2000), which leads to a progressive decrease of pO_2 and increase of pCO_2 in the PVF along with the growth of the embryo (Gutowska and Melzner, 2009). Under acidified conditions, the pCO_2 (and therefore the H^+ concentration) in the PVF is likely raised as

observed in the cuttlefish eggs (*i.e.* pCO_2 in PVF were 5-, 4- and 3-fold higher than in seawater, *i.e.* at 380, 850 and 1500 µatm, respectively, unpubl. data). As observed in the squid egg strands and shells, this increased H^+ concentration in the PVF might lower accumulation / adsorption of both elements in embryo. A not-restrictive but alternative explanation is that, in such conditions of hypoxia and hypercapnia within the egg, squid late embryos exhibit a metabolic depression (Guppy and Withers, 1999) - 2-fold lower oxygen consumption compared to the new hatchlings (Melzner et al., sbm) - that could be enhanced with increasing seawater pCO_2 (Rosa and Seibel, 2008). This may therefore contribute to limit the accumulation of metals such as Cd and Mn in their tissues.

Secondly, regarding ^{110m}Ag, ^{57}Co, ^{203}Hg and ^{65}Zn, it is noteworthy that the more the metal is retained by the eggshell, the lower is the penetration in the embryo. More precisely, the behaviours of metals that are mainly retained by the external part of the eggshell (*i.e.* ^{203}Hg and ^{65}Zn; Table 2), are particularly affected by pCO_2. The Co, mainly associated with the inner layers of the eggshell (Table 2), showed an opposite behaviour with a limited penetration in the embryo (lowest CF values; Table 2) unaffected by the seawater pCO_2. This result support the view that pCO_2-induced modifications of metal binding and shielding properties of the external eggshell layers have a direct impact on the Ag, Hg and Zn accumulation in the embryo that are mainly retained by these nidamental envelopes.

Following hatching, exposure of eggshell-free paralarvae to waterborne ^{110m}Ag, ^{203}Hg and ^{65}Zn under hypercapnic conditions (Fig.3) reveals that pCO_2 affects directly and significantly the CF values in squid early-life stages. Comparison of metal uptake in embryo and juvenile raises some hypotheses about the impact of the pCO_2 on the mechanisms of Hg, Zn and Ag accumulation in these organisms.

More precisely, Hg uptake in paralarvae was enhanced at 850 µatm compared to the control and the highest pCO_2 value, as previously observed on the whole egg strand. In cuttlefish juveniles, ^{203}Hg is mainly stored (up to 80% of the total Hg load) in muscles, skin and gills. This suggests that this metal is mainly bound to tissues directly exposed to contaminated seawater (Lacoue-Labarthe et al., 2009b) and that intermediate pCO_2 could favour this Hg adsorption to the surface tissues of the paralarvae. However, further studies should be carried out, for instance using autoradiography, to confirm this hypothesised localisation of Hg ions in the cephalopod juveniles.

The bioaccumulation rate of ^{65}Zn in the paralarvae was not affected by the pCO_2 treatments (Fig. 3), although it was still enhanced by increasing pCO_2 in the embryo at the

end of development (Fig. 2). This enhancement was also observed in the statoliths. This suggests that the incorporation of Zn in the statolith is proportional to the quantity of Zn bioaccumulated in the embryo. Alternatively, the increasing Zn incorporation in statoliths could be linked with increased growth of this calcified structure under low pH conditions as observed in fish otoliths (Checkley et al., 2009) and Sepioid cuttlebone (Gutowska et al., 2010a). This observation suggests that ocean acidification affects the incorporation rate of a metal that has been used as an environmental tracer in cephalopods (Ikeda et al. 1996, 2002; Lipinski et al. 1997; Zumholz et al. 2007) and hopefully will stimulate future research to investigate the possibility it could impact the incorporation of other trace elements used to track movements of marine organisms (Arkhipkin et al. 2004; Campana et al. 2000). Furthermore, Zn is a co-factor of carbonic anhydrase enzyme that is strongly involved in acid-base regulation (Addink, 1971) and therefore in compensation to hypercapnia-induced acidosis (Pörtner et al., 1989; Gutowska et al., 2010b). In this context, the high hypercapnia experienced by the late embryo in the PVF might enhance carbonic anhydrase synthesis and Zn requirements in the tissues. Further evaluation is now required to evaluate this hypothesis that increased Zn accumulation in organisms is related to pCO_2-compensation in their physiological processes.

Acid-base regulation is coupled to ionic regulation as the transfers of H^+ and HCO_3^- across biological membranes require an exchange with Na^+ and Cl^- (*e.g.* Gilmour and Perry, 2009). In these processes, the Na^+/K^+-ATPase is thought to be the main mechanism of ion exchange by creating ion- and electrochemical gradients used by secondary active transporters (Evans et al. 2005; Gilmour and Perry, 2009). In marine organisms, the active uptake of sodium is the mechanism by which Ag penetrates across the gills (Webb and Wood, 2000; Grosell and Wood, 2001) and the inhibition of Na^+/K^+-ATPase is key to Ag toxicity (Bianchini et al., 2005). In a recent study, Hu et al. (2010) showed that the squid embryo and paralarvae display relatively low gill ion-regulatory capacities linked with less developed gill epithelia compared to the cuttlefish. However, both the normal development of squid and cuttlefish embryos under extreme abiotic conditions in the PVF (Melzner et al., sbm) and the higher Ag CF values reached in squid hatchlings (^{110m}Ag CF = 2025 ± 176 and 5537 ± 2840 in late stages embryos of cuttlefish and squid, respectively; Lacoue-Labarthe, sbm) suggest that another organ, *e.g.* skin or the yolk-sac membrane, might perform the ion-regulatory functions in squid embryo (Hu et al., 2010). Thus, the increased Ag uptake with increasing pCO_2 found in the present study both in the embryo and the paralarvae seems to result from

enhanced compensation processes involving ionic regulation mechanisms, in response to hypercapnia.

In summary, this study confirms the appreciable and contrasting effects of pCO_2 on the bioaccumulation of several metals at different early-life stages of the squid *Loligo vulgaris*. By comparing the uptake of multiple radiotracers between enclosed embryos and eggshell-free paralarvae, we hypothesize the following; i) the ocean acidification leads to combined effects of pH / protons on the binding efficiencies of biological surfaces and consequently on the protective properties of the eggshell (and especially the external envelopes), and ii) the hypercapnia affects the accumulation of metals in the biological tissues through disturbances to physiological processes of the organism. In the context of the ocean acidification, these results improve our knowledge of the ecotoxicological consequences of reduced or increased accumulation of essential and non-essential metals on the survival and recruitment success of early-life stages.

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Table 1. *Loligo vulgaris*. Parameters of ^{110m}Ag, ^{109}Cd, ^{57}Co, ^{203}Hg, ^{54}Mn, and ^{65}Zn uptake kinetics in the squid egg strands (n = 4) and paralarvae (n = 5 pools of 5-10 individuals) exposed for the whole development time and for 52 h, respectively, to radiotracers dissolved in seawater in three pH levels treatments (see Figure 1 and 3):

			Uptake kinetics in egg strands					**Uptake kinetics in paralarvae**			
Metal	pH	Model	k_u (d^{-1})	CF_{ss} ± SE	k_e (d^{-1})	R^2	Model	k_u (d^{-1})	CF_{ss} ± SE	k_e (d^{-1})	R^2
Ag	7.60	C	0.050* (a)	161 ± 33 (a)	0.260**	0.943	E	-	2382 ± 68 (a)	0.186***	0.960
	7.85	C	0.078*** (b)	152 ± 17 (a)	0.295***	0.985	E	-	1948 ± 116 (b)	0.133***	0.863
	8.10	C	0.045** (a)	159 ± 24 (a)	0.249***	0.977	E	-	1357 ± 67 (c)	0.379*	0.893
Cd	7.60	E	-	37 ± 4 (a)	0.112***	0.722	E	-	-	-	
	7.85	E	-	29 ± 2 (b)	0.310***	0.595	E	-	-	-	
	8.10	E	-	77 ± 5 (c)	0.164***	0.842	L	-	-	-	-
Co	7.60	L	4.488*** (a)	-	-	0.970	E	-	7 ± 1 (a)	0.051**	0.879
	7.85	L	4.942*** (a)	-	-	0.979	E	-	15 ± 5 (ab)	0.027^{ns}	0.807
	8.10	L	4.978*** (a)	-	-	0.941	E	-	18 ± 5 (b)	0.018*	0.943
Hg	7.60	E	-	334 ± 9 (a)	0.248***	0.902	L	1.573*** (a)	-	-	0.912
	7.85	E	-	371 ± 9 (b)	0.295***	0.892	L	2.222*** (b)	-	-	0.964
	8.10	E	-	292 ± 8 (c)	0.247***	0.922	L	1.448*** (a)	-	-	0.938
Mn	7.60	E	-	50 ± 3 (a)	0.180***	0.819	L	0.455*** (a)	-	-	0.975
	7.85	E	-	78 ± 6 (b)	0.093***	0.886	L	0.536*** (a)	-	-	0.961
	8.10	E	-	113 ± 5 (c)	0.105***	0.966	L	0.518*** (a)	-	-	0.974
Zn	7.60	E	-	201 ± 18 (a)	0.076***	0.914	L	0.748*** (a)	-	-	0.965
	7.85	E	-	208 ± 10 (a)	0.083***	0.962	L	0.979*** (a)	-	-	0.940
	8.10	E	-	299 ± 16 (b)	0.074***	0.978	L	0.848*** (a)	-	-	0.896

L, E and C: linear, exponential and combined models, respectively; CF_{ss}: concentration factor at steady-state, k_u and k_e: uptake and elimination rate, respectively; SE: standard error; R^2: determination coefficient; p-values: < 0.001 (***), < 0.01 (**), < 0.05 (*), > 0.5 (ns). Different letters denote statistically significant differences (F test; $p < 0.05$) between the sample pH.

Table 2. *Loligo vulgaris*. Radiotracers distribution (%; mean ± SD, n = 3) and CF in brackets (mean ± SD, n = 3) in the different compartments of the squid egg strand (the eggshell from which external and internal layers were separated, embryos, and the perivitelline fluid) at the end of development (stage 29-30) after a whole development time exposure to radiotracers dissolved in seawater in three pH levels treatments.

	^{110m}Ag			^{109}Cd			^{57}Co		
	7.60	7.85	8.10	7.60	7.85	8.10	7.60	7.85	8.10
Eggshell	37.3 ± 12.4 (187 ± 58)^{c}	52.4 ± 6.5 (425 ± 102)^{b}	54.5 ± 16.2 (255 ± 74)^{a}	92.9 ± 3.2 (63 ± 20)^{a}	92.4 ± 3.6 (86 ± 27)^{a}	92.7 ± 4.4 (165 ± 58)^{b}	91.8 ± 4.7 (211 ± 39)a	92.5 ± 2.0 (246 ± 34)a	90.3 ± 11.2 (214 ± 104)a
External part	18.9 ± 10.4	22.6 ± 8.4	35.4 ± 12.8	40.1 ± 20.1	68.8 ± 7.5	54.7 ± 16.4	22.7 ± 10.5	20.6 ± 4.7	23.5 ± 12.1
Internal part	18.4 ± 6.8	29.9 ± 8.0	19.1 ± 8.9	52.8 ± 18.5	23.7 ± 5.9	38.0 ± 15.6	69.1 ± 9.3	71.9 ± 5.2	66.6 ± 21.5
Embryo	61.8 ± 12.5 (10200 ± 1350)^{b}	46.9 ± 6.8 (8587 ± 43)^{a}	44.9 ± 16.2 (9341 ± 457)^{a}	5.2 ± 2.8 (109 ± 52)^{b}	6.8 ± 3.8 (142 ± 83)^{b}	6.8 ± 4.3 (505 ± 299)^{a}	0.9 ± 0.2 (63 ± 16)^{a}	1.0 ± 0.4 (67 ± 37)^{a}	7.7 ± 10.6 (347 ± 378)^{a}
Perivitelline fluid	1.0 ± 0.7 (32 ± 10)	0.7 ± 0.4 (43 ± 33)	0.6 ± 0.5 (60 ± 86)	2.2 ± 1.7 (< 15)	< 1 (< 5)	< 1 (< 5)	8.3 ± 4.3 (139 ± 29)	6.4 ± 2.0 (126 ± 13)	2.3 ± 1.7 (88 ± 48)

	^{203}Hg			^{54}Mn			^{65}Zn		
	7.60	7.85	8.10	7.60	7.85	8.10	7.60	7.85	8.10
Eggshell	97.3 ± 1.2 (959 ± 129)^{ab}	98.3 ± 0.3 (1088 ± 102)^{b}	98.1 ± 1.5 (935 ± 129)^{a}	96.8 ± 0.9 (102 ± 15)^{b}	98.2 ± 0.6 (177 ± 49)^{a}	96.0 ± 2.4 (201 ± 50)^{a}	70.7 ± 8.4 (199 ± 36)^{c}	72.9 ± 7.0 (285 ± 86)^{b}	90.3 ± 5.2 (422 ± 108)^{a}
External part	84.4 ± 6.6	85.6 ± 8.2	92.3 ± 3.4	64.7 ± 11.4	69.8 ± 10.0	80.2 ± 6.4	35.4 ± 14.0	54.1 ± 13.7	66.0 ± 12.8
Internal part	12.9 ± 5.9	12.7 ± 8.3	5.8 ± 3.2	32.0 ± 10.6	28.4 ± 9.6	15.2 ± 5.7	35.3 ± 8.3	18.8 ± 7.9	24.0 ± 12.6
Embryo	2.5 ± 1.2 (752 ± 112)^{a}	1.5 ± 0.2 (394 ± 93)^{b}	1.8 ± 0.1 (662 ± 326)^{a}	3.1 ± 1.0 (103 ± 20)^{b}	1.8 ± 0.6 (75 ± 37)^{b}	4.0 ± 2.4 (311 ± 70)^{a}	29.1 ± 8.4 (2613 ± 398)^{b}	26.9 ± 7.0 (2337 ± 733)^{ab}	9.9 ± 5.2 (1819 ± 183)^{a}
Perivitelline fluid	0.2 ± 0.1 (17 ± 4)	0.2 ± 0.1 (14 ± 2)	1.5 ± 0.1 (22 ± 32)	0.2 ± 0.1 (< 5)	0.1 ± 0.02 (< 5)	0.1 ± 0.1 (< 5)	0.2 ± 0.1 (< 5)	0.2 ± 0.1 (< 5)	0.1 ± 0.1 (<5)

Different letters denote statistically significant differences (Kruskall-Wallis test; $p < 0.05$) between the sample pH.

Figures captions

Fig. 1. *Loligo vulgaris.* ^{110m}Ag, ^{109}Cd, ^{57}Co, ^{203}Hg, ^{54}Mn and ^{65}Zn uptake kinetics (CF; mean ± SD; n = 4) in the whole egg strands exposed to dissolved radiotracers at three different pH – pH 8.10 (l), pH 7.85 (p), pH 7.60 (▲).

Fig. 2. *Loligo vulgaris*. Concentration factors of ^{110m}Ag, ^{109}Cd, ^{57}Co, ^{203}Hg, ^{54}Mn and ^{65}Zn (CF; n = 9 pools of 7-26 embryos from 3 egg strands), in the squid embryos exposed dissolved radiotracers at three different pH – pH 8.10, pH 7.85, pH 7.60 – for two development times, i.e. 16 d (stage 25-26; grey) and 26 d (stage 29-30; white). Results of the statistical analysis were reported on the Table 2.

Fig. 3. *Loligo vulgaris.* ^{110m}Ag, ^{57}Co, ^{203}Hg, ^{54}Mn and ^{65}Zn uptake kinetics (CF; mean ± SD; n = 5 pools of 5-10 individuals) in the squid paralarvae exposed to dissolved radiotracers at three different pH – pH 8.10 (l), pH 7.85 (p), pH 7.60 (▲).

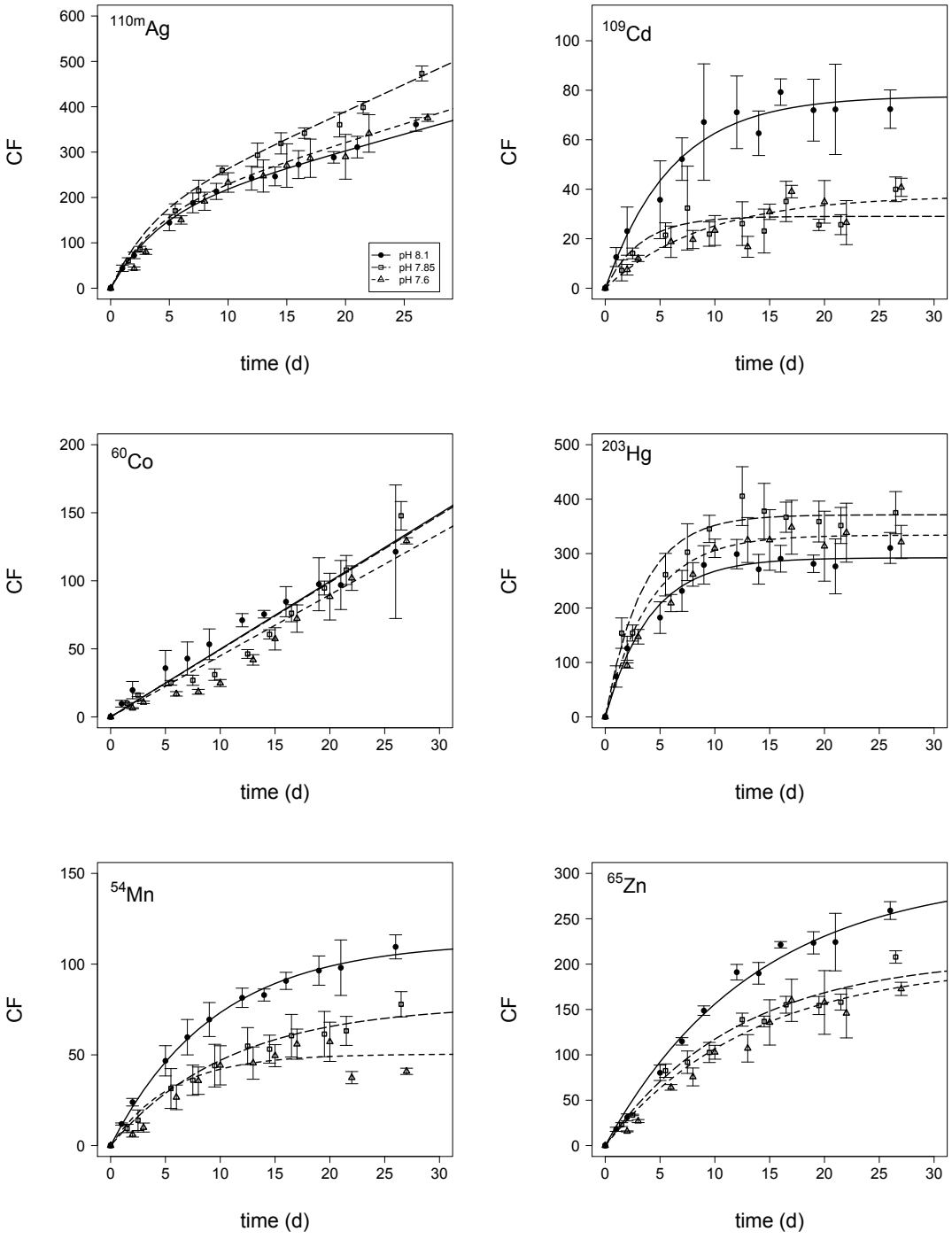

Fig. 1.

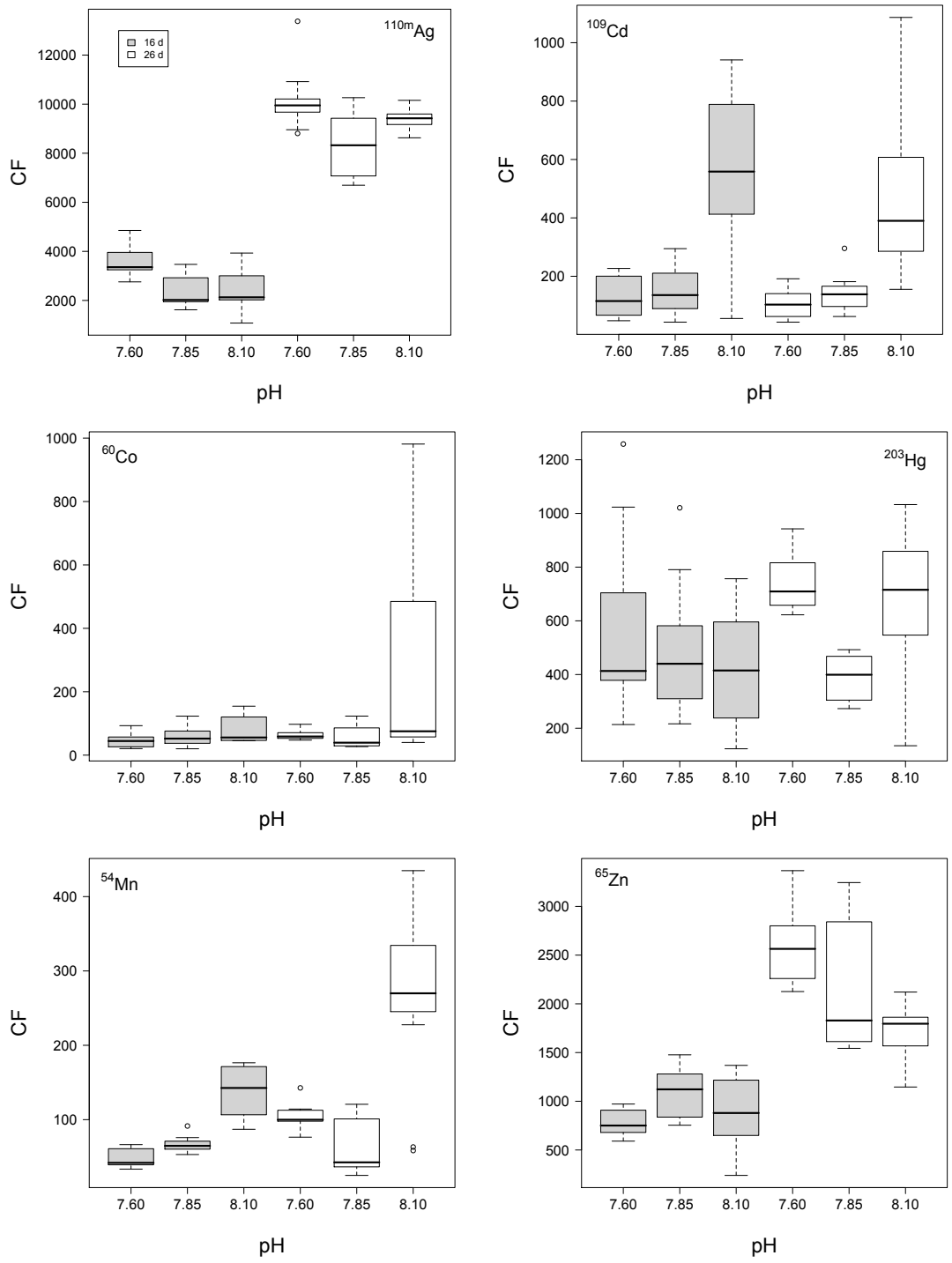

Fig. 2.

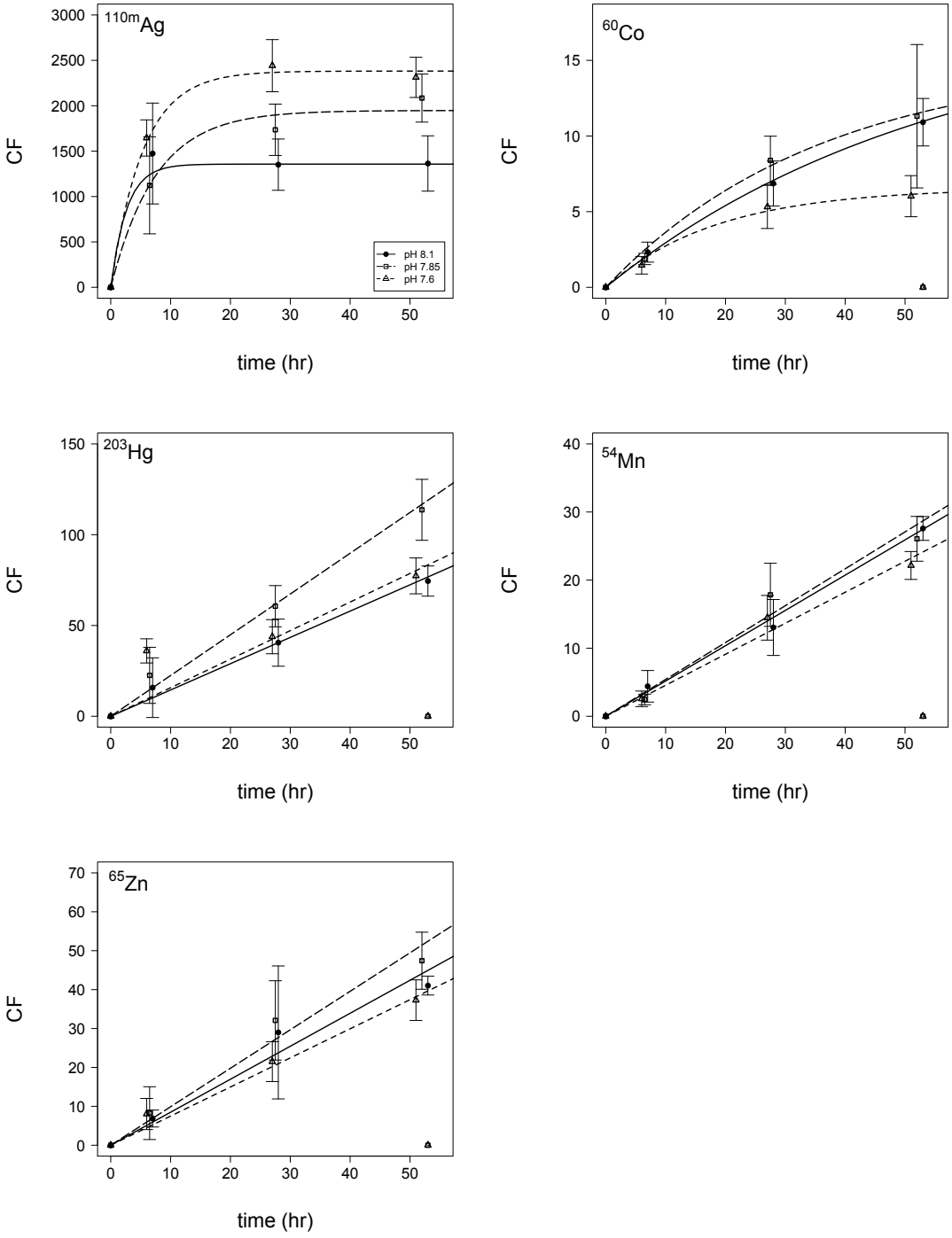

Fig. 3.